Tumor Necrosis Factor Alpha is increased after cavernous nerve injury and impairs regeneration of nitrergic neurons and modulates penile smooth muscle tone

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Introduction

Despite the establishment of nerve-sparing radical prostatectomy (RP), erectile dysfunction (ED) is still a major complication after RP. A cause of ED following RP is cavernous nerve (CN) injury at the time of surgery [Walsh. *J Urol* 2007, *Urology* 2000].

Generally, peripheral nerve injury (PNI) activates Schwann cells, which secrete inflammatory cytokines and chemokines to recruit macrophages [Smith. *J Neurosci Res.* 1998].

Previously, we demonstrated that increase in inflammatory cells, particularly neurotoxic M1 macrophages, can lead to impaired smooth muscle relaxation following bilateral CN injury (BCNI) [Matsui, *et al*. *JSM 2017*]. Tumor necrosis factor alpha (TNFA) is one of the cytokines secreted both by Schwann cells and macrophages.

The net result of increased TNFA was shown to induce neuronal cell death following sciatic nerve injury [Shamash. *J Neurosci* 2002].

In the major pelvic ganglion (MPG), the gene expression of TNFA was increased at BCNI [Matsui, *et al*. *JSM 2017*].

Objectives

The aim of this study is to 1) examine temporal changes of TNFA, after BCNI in vivo and 2) its effect on ex vivo neurite outgrowth from MPG with exogenous administration. In addition, 3) we examined effect of TNFA signal inhibition on penile smooth muscle function ex vivo with TNFA receptor -1,2 knockout mice (TNFR-KO).

Methods

Study 1 *in vivo*

- Male Sprague-Dawley rats (12 weeks, 300-350g)
- Rats were randomized to undergo BCNI or sham surgery. Sham rats' MPGs were harvested after 48 hours. MPGs of BCNI groups were harvest at 6 hours, 12 hours, 24 hours, 48 hours, 7 days, or 14 days after surgery (5 rats/group).
- Western blot (WB) was used to evaluate protein amount of TNFA in MPGs, and immunofluorescence (IF) was used to localize TNFA.

Methods

Study 2 *ex vivo*

- Whole MPGs were harvested from non-crushed rats and cultured in Matrigel with TNFA in concentrations of 0, 10, 20, 30 ng/mL (n=5) [Montrucchio. *JEM* 1994].
- Neurites were measured at 48 and 72 hours after culture. Average lengths of 5 longest neurites in each area were compared. . [Matsui. Urology. 2017].
- MPGs were processed for qPCR 72 hours after culture. [Hannan. J Neurosci Res 2015].
- Additional MPGs were cultured with or without TNFA 20 ng/mL for IF TH and nNOS.

Study 3 *TNFRKO*

- Wild type (WT) and TNFR-KO mice underwent either Sham or BCNI (n=5/group)
- MPGs were collected 48 hours after surgery and processed for qPCR to evaluate gene expression of nNOS. neuronal nitric oxide synthase (nNOS), tyrosine hydroxylase (TH).
- Penises were harvested to evaluate smooth muscle function to electrical field stimulation (EFS) with myography.

Results: *in vivo* Study

BCNI increased protein expression of TNFA in MPGs

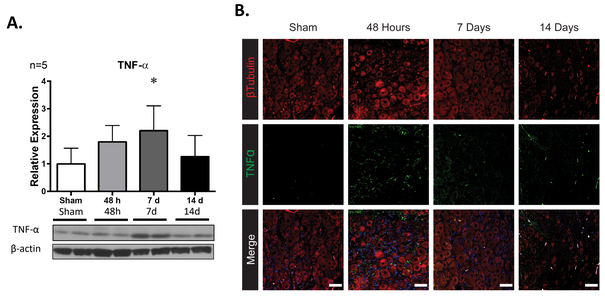

Figure 1. (**A**) WB analysis of TNF-α and β-actin in MPGs at 48 hours, 7 days, and 14 days after BCNI. Relative protein amount of TNF-α to β-actin is indicated in the bar graphs. * indicates p<0.05 compared to Sham. Representative WB of TNF-α and β-actin is also demonstrated.

(**B**) Representative immunofluorescences of TNF-α and β-tubulin in the MPGs of sham rats and MPGs harvested from BCNI rats at 48 hours, 7 days, and 14 days after surgery. TNF-α was primarily detected in the perivascular area at 48 hours, around the cell bodies of the neurons, and cytoplasm of the cell bodies at 14 days

Results: *ex vivo* Study

Exogenous TNFA Impaired Neurite Outgrowth from the MPG in a Dose-Dependent Fashion

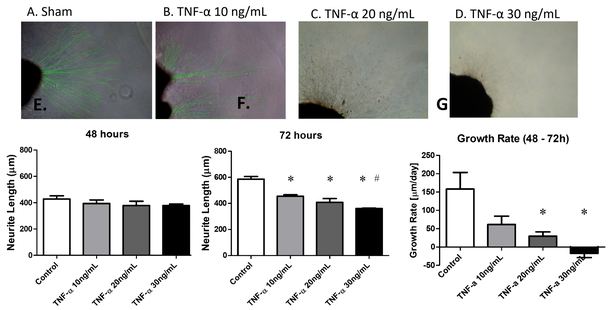

Figure 2. Panels A-D show representative images of neurite outgrowth from MPGs of control, MPGs cultured with exogenous TNFA at concentrations of 10, 20, and 30 ng/mL. Panel E shows bar graphs of neurite lengths 48 hour after culture and panel F shows bar graphs of neurite lengths 72 hours after culture. * indicates p<0.05 compared to control. Panel G demonstrates growth rates at 48-72 hour after culture at each concentration of exogenous TNFA. * indicates p<0.05 compared to control.

TNFA Group Displayed Bimodal Distribution of Neurite Lengths

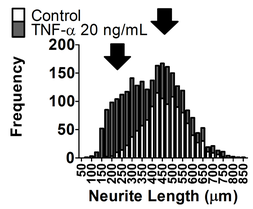

Figure 3. Histogram of neurite lengths. Control group displayed normal distribution, while TNF-α group had bimodal distribution: One peak at 400 μm and the other at 275 μm. These results suggest that some neurites are not affected by exogenous TNF-α.

Neurite Outgrowth of Nitrergic Neurons Was Significantly Inhibited by Exogenous TNF-α

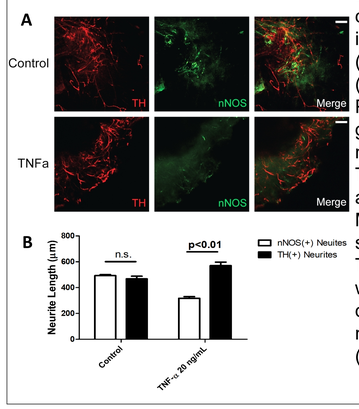

Figure 4. Panel A demonstrates representative immunofluorescences of TH (stained in red) and nNOS (stained in green). Panel B demonstrates bar graphs of neurite lengths of nNOS positive neurons and TH-positive neurons in control and TNF-α 20 ng/mL groups. MPGs cultured with TNFA had shorter nNOS+ neurites than TH+ neurites (p<0.01), whereas there was no difference in nNOS and TH+ neurite lengths in controls (p=0.29).

Results: TNFRKO Study

Gene expression of nNOS was enhanced in TNFRKO

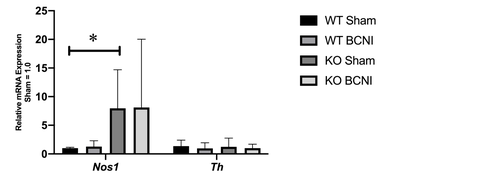

Figure 5. (A) Bar graphs of the relative gene expression of Nos1 and Th in WT sham, WT BCNI, TNFRKO sham, and TNFRKO BCNI (n=5/group). * indicates p<0.05 compared to WT sham group. Gene expression of nNOS was upregulated in TNFRKO compared to WT (p<0.01).

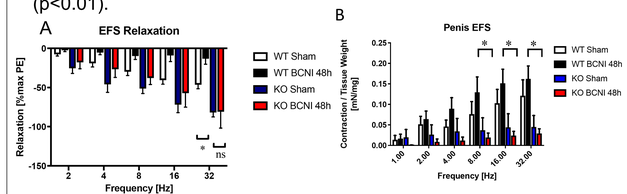

Figure 6. (A) Smooth muscle relaxation response and (B) contractile response to EFS, respectively. Pre-contraction was induced with phenylephrine. * indicates p<0.05 compared to WT sham group.

TNFKO demonstrated enhanced penile relaxation response to EFS following maximal contraction with phenylephrine compared to WT (p<0.01). Penile smooth muscle relaxation was impaired in WT after BCNI (p<0.01) but this change was not seen in TNFRKO.

Penises from WT demonstrated enhanced smooth muscle contraction to EFS compared to TNFKO (p<0.01).

Conclusions

This study demonstrates that TNF-α is increased after BCNI and exogenous TNF-α impairs neurite outgrowth from MPGs in a dose-dependent fashion.

Furthermore, exogenous TNF-α downregulated gene and protein expression of nNOS.

TNFRKO mice demonstrated enhanced gene expression of nNOS and enhanced penile smooth muscle relaxation.

These results indicate that TNF-α selectively inhibits regeneration of nitrergic neurons and TNF-α inhibition may prevent ED after BCNI by protecting nitrergic nerves.

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